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Angiotensin inhibition reduces glomerular damage and renal chemokine expression in MRL/lpr mice

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Non-Standard Abbreviations:

RAS: Renin-Angiotensin-System; BP: Mean arterial blood pressure; ACEI: Angiotensin-converting-enzyme inhibitor; CCL: CC chemokine ligand, CXCL: CXC chemokine ligand.

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Abstract

Beneficial effects of angiotensin II inhibition during inflammatory renal disease may involve both hemodynamic and non-hemodynamic mechanisms. To analyse whether angiotensin II inhibition has protective effects on lupus-like, autoimmune-mediated renal damage in MRL/lpr mice, 4 groups of mice were treated orally for 6 weeks with: 1) vehicle, 2) enalapril (3.0 mg/kg day), 3) candesartan cilexetil (5.0 mg/kg) or 4) amlodipin (10 mg/kg) as a blood pressure control (n=9-12 per group). All antihypertensive treatments lowered blood pressure to a similar level as compared to vehicle group (enalapril: 99.8±8.3 mmHg; candesartan: 101±9; amlodipin: 103.8±6.7; vehicle: 113.5±4.6). Vehicle treated mice developed a moderate glomerular injury with albuminuria (35.1±39.0 μg/mg creatinine). Glomerular lesions consisted of immune complex deposition and mesangial expansion with increased mesangial cell proliferation. Amlodipin treatment had no significant protective effects. In contrast to vehicle and amlodipin treated mice, those subjected to angiotensin II blockade with enalapril or candesartan had reduced albuminuria, glomerular expansion and mesangial proliferation. This was associated with significantly reduced renal chemokine mRNA expression as compared to vehicle treatment. Our results show that inhibition of angiotensin II has protective effects on the glomerular damage of MRL/lpr mice that extend beyond hemodynamics and involve down-modulation of glomerular inflammation, reduction of mesangial cell proliferation and decrease in chemokine expression.

The role of the renin-angiotensin system (RAS) in the progression of experimental renal disease (Mackenzie et al., 1994; Wolf et al., 1997; Ruiz-Ortega et al., 1998; Hisada et al., 1999; Satoh et al., 2001) and in patients with various forms of renal disease (Ravid et al., 1993; Burnier and Brunner, 2000) is well established. Increased expression of mRNA for components of the RAS (renin, ACE and angiotensinogen) has been shown in kidneys of nephritic and hypertensive patients (Lai et al., 1998). Their findings support the notion that among other diseases, in immune-complex mediated glomerulonephritis the intrarenal activation of the RAS may be of pathogenic relevance. The beneficial effect of blocking angiotensin II generation or its receptors appears to involve both hemodynamic (Navar et al., 1996; Kim and Iwao, 2000) and non hemodynamic, cytokine-mediated mechanisms (Hisada et al., 1999; Nataraj et al., 1999).

Recent evidences points towards additional effects of RAS inhibition to influence immunological factors such as chemokines. For example, angiotensin converting enzyme (ACE) inhibition results in a reduction of proteinuria and renal tissue damage through a reduction of CCL2/MCP-1 synthesis in immune complex nephritis in rats (Ruiz-Ortega et al., 1998). Both treatments with ACEI and AT1 receptor antagonists reduced CCL2/MCP-1 mRNA synthesis in unilaterally urether ligated rats (Morrissey and Klahr, 1998) and in a model of diabetic nephropathy in rats (Kato et al., 1999). In addition, AT1a deficient mice were protected against anti-glomerular basement membrane nephritis as compared to wild-type control mice and showed less CCL2/MCP-1 and TGF-ß production (Hisada et al., 1999). Finally, *in vitro* studies have shown the influences of angiotensin II on cellular immune responses through a calcineurin-dependent pathway (Nataraj et al., 1999).

The aim of the present work was to study effects of angiotensin II blockade on the immune-complex-mediated renal damage in MRL/lpr mice. These mice develop a spontaneous autoimmune disease, which shows similarities to human systemic lupus erythematosus

(Theofilopoulos and Dixon, 1981). The apoptosis-related fas gene in this mouse strain is modified carrying the mutation called *lpr*, which results in an aberrant transcript and a non-functional protein (Adachi et al., 1993). Autoreactive lymphocytes escape thymic selection (Merino et al., 1993), start their proliferation and produce antibodies against own cell structures. As a consequence, circulating immune complexes are produced and deposited in the renal glomerular microvasculature. There, immune complexes trigger the synthesis of various mediators of inflammation, resulting in cellular infiltration, proteinuria, and progressive renal failure. In previous studies we and others demonstrated that chemokine expression appears as an early molecular process contributing to the inflammatory process of lupus nephritis in mice (Zoja et al., 1997; Tesch et al., 1999; Pérez de Lema et al., 2001). Similar observations on the roles of chemokines have been reported for patients with lupus nephritis (Rovin et al., 1994; Wada et al., 1996). In addition, CCL2/MCP-1-deficient MRL/lpr mice show a reduction in renal mononuclear cell infiltrates and proteinuria leading to prolonged survival as compared to wild-type or heterozygous lupus mice (Tesch et al., 1999). In the present study we therefore tested the hypothesis that inhibition of the angiotensin II system would ameliorate renal inflammation in MRL/lpr mice by reducing the expression of chemokines. By contrast comparable reduction in mean arterial blood pressure (BP) by the calcium antagonist amlodipin might not have such a protective effect, a hypothesis supported by our results.

Methods

Reagents and Antibodies

The following antibodies were used: a rabbit anti-mouse Ki67 antiserum (Dianova, Hamburg, Germany); a FITC-conjugated rabbit anti-mouse Ig antibody (Boehringer Mannheim, Mannheim, Germany) and a peroxidase-conjugated goat anti-rabbit Ig secondary antibody (Dako, Glostrup, Denmark). All reagents for the anti-DNA enzyme-linked immunosorbent assay (ELISA) were from Boehringer Mannheim, except the mouse monoclonal anti-single- and double-stranded DNA antibody used as a standard (Chemicon International, Temecula, CA). Radiolabeled [α-^{32}P]-UTP (3000 Ci/mmol) for RNase Protection Assay was from NEN (Boston, MA). All other reagents or solvents (analysis grade) were from Merck (Darmstadt, Germany). The multiprobe ribonuclease protection assay (RPA) template set mCK5 lacking the IP-10 probe was from Pharmingen (San Diego, CA). Polyethyleneglycol 6000 at 0.1% wt/wt, 0.1% ethanol (wt/vol), 2.0 mM $NaHCO_3$ in aqua ad injectabila served as vehicle for the administration of the drugs (Mackenzie et al., 1994). Candesartan cilexetil (candesartan) drug was obtained from Takeda Chemical Industries, Ltd. (Osaka, Japan). Xanef injectable solution (1 mg/ml) from MSD and Norvasc from Pfizer were the galenic forms used for the enalapril and amlodipin treatments, respectively.

Experimental Design

MRL/MpJ-Tnfrsf6^{lpr} (MRL/lpr) mouse line was obtained from The Jackson Laboratory (Bar Harbor, ME) and bred and housed under specific pathogen-free conditions, with free access to water and food. All animal experimental procedures were performed in Germany after authorisation by the local ethical committee according to German law (211-2531-83/2000). Female virgin MRL/lpr mice were randomly distributed into the different experimental groups and kept individually. Beginning at the age of 8 wk, mice were treated (by adding the drug

daily to 5 ml drinking water) with a) vehicle; b) enalapril (3.0 mg/kg body weight); c) candesartan (5.0 mg/kg); d) amlodipin (10.0 mg/kg). These dosages are within the usual therapeutic ranges used in mice (Tarkowski et al., 1990; Traynor and Schnermann, 1999). The completeness of the treatment was assessed daily by measuring the amount of fluid consumed. Mice showing irregular intake and those who took less than 80% of the mean dose were rejected for further analysis. For each group, 9-12 mice were finally analysed. Spot urine and blood samples were obtained every two weeks. The following parameters were determined using standard analytical protocols: albuminuria assessment by ELISA (Bethyl Laboratories, TX, USA), Jaffee method (Bartels et al., 1972) for creatinine measurements (Merck Diagnostika), urease/glutamate dehydrogenase method (Hoffmann, 1971) for blood urea nitrogen measurements (Merck Diagnostika), IgG ELISA for analysis of circulating IgG (Boehringer Mannheim) and anti-double-stranded DNA antibodies (Pisetsky and Peters, 1981).

After BP measurement (see below) mice were sacrificed. One kidney was used exclusively for isolation of total RNA, using a standard protocol (Chomczynski and Sacchi, 1987). The other kidney was split into two halves. One half was fixed in formaldehyde for routine histological examination or immunohistological studies. The other half of the renal tissue was embedded in OCT medium (Jung; Leica Instruments, Wetzlar, Germany), snap-frozen in liquid nitrogen, and stored at -80°C until used for immunohistological studies.

Measurement of blood pressure

At the age of 14 weeks, BP was determined as previously described (Stauss et al., 1999). Mice were anesthetised by a single intraperitoneal injection of fentanyl, midazolam, and medetomidin (0.05, 5.0 and 0.5 mg/kg body weight, respectively). The left femoral artery was exposed and cannulated by a polyethylene tube. The arterial catheter was connected to a pressure transducer located at the same level as the mice (Statham, Costa Mesa, CA) via a

swivel device (Instech Laboratories Inc., Plymouth Meeting, PA, USA). After surgery naloxon, flumazenil, and atipamezol (1.2, 0.5, and 2.5 mg/kg body weight, respectively) were injected s.c. to antagonize the effect of the anesthetic drug. Thus, the mice recovered from anesthesia within 5 to 10 min. In pilot experiments we found that arterial pressure stabilised within 90 min and did not change significantly in the next 6 hours. Therefore pressure was determined continuously for 60 min starting 90 min after injection of the antagonists. The pressure signal was processed with a computer based monitoring system (XmAD, ftp://sunsite.unc.edu/pub/Linux/science/lab) at a sampling rate of 1000 Hz. Heart rate was calculated by analyzing software (XmANA) from the amplitude of the pressure signal.

Light Microscopy

Renal tissue was fixed in neutral buffered formaldehyde in saline, and 3-4 µm, paraffin-embedded sections were stained with hematoxylin & eosin (H&E) and periodic acid-Schiff stain (PAS). Glomerular injury was semiquantified by a renal pathologist blinded for the treatments considering glomerular hypercellularity, leukocyte exudation and mesangial matrix expansion. Each of these parameters was graded as 0 (absence), 1+ (mild), 2+ (moderate) or 3+ (severe), and a glomerular index defined as the simple sum of all values. Interstitial cell infiltrates affecting the renal cortex and with a peritubular and pericapillar distribution were similarly graded as 0 to 3+.

Immunohistochemistry

Studies were performed on frozen tissue sections and processed as described above, using standard techniques (Mampaso and Wilson, 1983). Direct immunofluorescence studies were performed on 5 µm, ether/ethanol-fixed, cryostat sections by using FITC-conjugated rabbit anti-mouse IgG antibody. Ki67-positive cells were characterised on paraffin embedded tissue 5-µm-thick sections from 4 independent mice per group. Peroxidase-conjugated goat anti-

rabbit IgG secondary antibody (Dako) was always developed with diaminobenzidine as chromogen. Respective preimmune sera or matched isotype IgG control served as negative controls. The quantification of Ki67 staining was performed by a pathologist blinded for the treatments by counting 50 glomeruli of 4 independent mice per group.

Renal mRNA Expression

Chemokine expression was analysed by a commercial ribonuclease protection assay (RPA) as previously described (Luckow et al., 2000). Twenty micrograms of total RNA from each sample was used. The RNase-protected probes were separated on 5% denaturing polyacrylamide gels and analysed by phosphorimaging (Storm 840 PhosphorImager; Molecular Dynamics, Sunnyvale, CA). Bands were quantified using ImageQuant software (Molecular Dynamics, Eugene, OR).

Statistical Analyses

Data were expressed as means ± standard deviation and analysed with either the unpaired two-way ANOVA and t-test with Bonferroni correction (for parametric data) or the Kruskal-Wallis and Mann-Whitney U test (for nonparametric data) as needed. The null hypothesis was rejected at $p<0.05$.

Results

Arterial blood pressure

In order to rule out different effects of ACEI or AT1-antagonists versus calcium antagonists, BP was determined after 6 weeks of treatment by direct arterial measurement at age 14 weeks. In MRL/lpr mice with progressive renal disease, treatment with amlodipin, enalapril or candesartan (n=5 in all groups) resulted in comparable and significant BP reduction as compared to vehicle-treated controls (fig. 1). No significant differences in the heart rate were observed between the groups (vehicle: 417±40 beats/min; enalapril: 433±18; candesartan: 413±35; amlodipin: 474±35) (n=5).

Plasma IgG and anti-DNA antibody levels

Total plasma IgG concentration increased with age in all groups. At the age of 14 weeks, no significant differences between enalapril-, candesartan-, amlodipin- or vehicle-treated MRL/lpr mice were observed (Fig. 2A). Similarly, plasma levels of anti-DNA antibodies (fig. 2B) were comparable in all groups at the age of 14 weeks of age.

Renal morphological findings

At the age of 8 weeks, when treatment was begun, no renal histological lesions were apparent (data not shown). At week 14 of age, untreated MRL/lpr mice showed well-established renal alterations in both glomerular and tubulointerstitial compartments (n=12). Glomerular lesions were characterised at this time point by enlarged, hypercellular glomeruli with increased numbers of both resident cells and infiltrating leukocytes, as well as mesangial matrix expansion (fig. 3A). Interstitial lesions included the presence of peritubular and pericapillar mononuclear cell infiltrates, which were focally and irregularly distributed through the whole cortex of the affected kidneys. As in human lupus, these lesions are very mild. By contrast to

these mild interstitial lesions, the presence of large accumulations of lymphoid cells located in the medulla and around big renal vessels is a hallmark of nephritis in MRL/lpr mice.

As shown in figures 3C and D, the development of glomerular lesions was reduced by 6 weeks of treatment with either enalapril (n=12) or candesartan (n=11), which reduced glomerular hypercellularity and mesangial matrix expansion as compared with age matched, vehicle treated MRL/lpr mice (fig. 3A). amlodipin treatment (n=10) had no significant effect on glomerular lesions (fig. 3B). Glomerular components of the renal disease were evaluated as described above. This glomerular injury score was significantly reduced by enalapril or candesartan, as compared to both vehicle and amlodipin control groups (fig. 4B).

In spite of the improvement of the glomerular lesions, none of the treatments had an effect on the lymphoproliferative interstitial findings (fig. 3 E and F).

Renal immune complex deposition

In order to examine whether differences in immune complex (IC) deposition might be a factor in the enalapril or candesartan-mediated effects, IC deposition was examined. At the age of 14 weeks, mice of all groups showed similar IgG-immune complex deposition patterns in the kidneys (n=3 in each group). IC deposits were generalised, diffuse, granular and irregularly distributed within the mesangium. No significant differences were observed in either the localisation or the intensity of the staining between the different treatment groups, (fig. 3 G-I). In some kidneys positive nuclear staining corresponding to anti-nuclear antibodies was observed. However, none of the treatments had any effect on this staining pattern.

Proteinuria and BUN levels

vehicle-treated MRL/lpr mice showed progressive increase in albuminuria from age 8 to 14 weeks. amlodipin treatment caused a slight non significant reduction of urinary albumin as

compared to vehicle treated mice. enalapril or candesartan treatment reduced albuminuria at week 14 compared to both vehicle and amlodipin-treated mice (fig. 4A) (n=10-12 per group). Blood urea nitrogen as a coarse measure of renal function was comparable in all groups (enalapril: 17.5±2.6; candesartan: 25.5±4.09; vehicle: 21.3±1.9 mg/dl; amlodipin: 23±2.9 mg/dl).

Mesangial proliferation

Mesangial expansion with increased cellularity and matrix was prominent in vehicle-treated, 14 weeks old MRL/lpr mice. enalapril and candesartan treatment resulted in marked reduction of mesangial proliferation. As a marker of proliferating cells we used the staining with Ki-67, which specifically recognises proliferating cells (Scholzen and Gerdes, 2000). Renal sections from 14 weeks old vehicle-treated mice showed prominent staining for the cell proliferation marker Ki-67. By contrast, both enalapril and candesartan treatments lead to a significant glomerular reduction of staining for the Ki-67 proliferation marker (Fig. 4C). Kidneys from amlodipin-treated mice showed Ki-67 staining comparable to the vehicle-treated group.

Chemokine expression

Our previous results had shown marked up-regulation of chemokine expression in kidneys from nephritic MRL/lpr mice (Pérez de Lema et al., 2001). To test the effect of the treatment, chemokine mRNA expression was analysed by RPA of total kidney RNA. As shown in figure 5, enalapril- or candesartan-treatment reduced the renal expression of the chemokines CCL2/MCP-1, CCL4/MIP-1ß and CXCL1/MIP-2 by 50-70% as compared to vehicle treated controls. This reduction was statistically significant for the case of the reduction of CCL2/MCP-1 expression after both enalapril and candesartan treatments and for CCL4/MIP-1ß in the candesartan group. The same trend was observed for the case of CXCL1/MIP-2 expression in both RAS blocking treatments and for CCL4/MIP-1ß in enalapril-treated mice,

but these differences did not reach statistical significance. By contrast, CCL5/RANTES expression was unaffected by RAS inhibition. As amlodipin had no effect on renal lesions and function, chemokines were not determined in this group.

Discussion

In the present work, we have studied the effects of the treatment with an ACE inhibitor (enalapril), an AT1-receptor antagonist (candesartan) or a calcium antagonist (amlodipin) on renal damage in a model of lupus-like disease in MRL/lpr mice. Based on previous experiments (Pérez de Lema et al., 2001) we chose the age of 8 weeks to start the treatment, as at that age no renal alterations are evident. A treatment period of 6 weeks was chosen, as at the age of 14 weeks well established renal lesions and increased proteinuria are observed (Pérez de Lema et al., 2001). Our results indicate, that the treatment with either enalapril 3 mg/kg or candesartan 5 mg/kg but not with amlodipin 10 mg/kg had a protective effect on renal, and specifically glomerular, damage. Both treatments having angiotensin II as a target resulted in a marked reduction of albuminuria as well as in glomerular renal damage as compared to that observed in either amlodipin or vehicle-treated mice. At week 14 of age, amlodipin or vehicle-treated mice showed proliferation and numerous Ki-67 positive cells in the glomeruli, which were markedly reduced in glomeruli of enalapril or candesartan treated kidneys. The presence of pertitubular and pericapillar mononuclear cell infiltrates was very mild and irregular at this age even in kidneys of vehicle-treated mice, arguing for a specific role of angiotensin II in the glomerular damage. The lymphoproliferative perivascular mononuclear cell infiltrates observed were not affected by angiotensin II-inhibition. This lesion is very characteristic of the MRL/lpr lupus model and consists mainly of lymphocytes (Carvalho-Pinto et al., 2002) and is most likely a sign of the lymphoproliferative phenotype as a consequence of the fas mutation rather than a consequence of any inflammatory response.

Our findings extend previous reports (Herlitz et al., 1988; Tarkowski et al., 1990) describing a protective effect of captopril (30 mg/kg) and no effect of enalapril (6.0 mg/kg) treatment on survival. According to these findings, both ACEI reduced glomerular damage in a similar

way, but only captopril significantly prolonged the survival. Surprisingly, while captopril reduced blood pressure at 14-17 weeks, as compared to vehicle treated controls, enalapril did not show this hypotensive effect at this age, whereas at later time points enalapril reduced blood pressure (Tarkowski et al., 1990). We observed a significant reduction of blood pressure at 14 weeks with enalapril. A possible explanation for this finding is that blood pressure was measured by tail plethysmography in Tarkowski's study, whereas we used direct intraarterial determination.

The renoprotective effects of angiotensin II blockade have been attributed to both hemodynamic-dependent and –independent mechanisms (Navar et al., 1996; Hisada et al., 1999; Nataraj et al., 1999; Kim and Iwao, 2000). In order to exclude effects of lowering the BP on the renal effects of the angiotensin II blockade we included amlodipin-treatment in an additional group of mice. The observed protection of angiotensin II inhibition on glomerular damage in MRL/lpr could not be attributed exclusively to a reduction in BP by enalapril or candesartan as amlodipin-treatment resulted in a comparable reduction in BP without a significant amelioration of albuminuria or histopathological glomerular damage. Obviously, this only excludes systemic BP as a factor, whereas intraglomerular hydrostatic pressure would be expected to be reduced by angiotensin II inhibition as compared with the treatment with a calcium antagonist (Zanchi et al., 1995). As we did not measure the glomerular intracapillar hydrostatic pressure a reduction in intraglomerular pressure by angiotensin II blockers remains a likely contributing factor for the beneficial effect observed. In fact, a decrease in intraglomerular hydrostatic pressure is considered a major renal therapeutic effect of angiotensin II blockers (Hollenberg, 2000).

Our experimental results strongly argue against differences in circulating IgG or anti-DNA antibodies, or their glomerular deposition as reasons for the mitigation of disease in ACEI- or AT-1 receptor antagonist-treated MRL/lpr mice. On the other hand we cannot exclude that

angiotensin II inhibition could affect the uptake of immune complexes. It is known, that angiotensin II enhances phagocytosis of immune complexes by mesangial cells and macrophages (Singhal et al., 1990). If this also occurs *in vivo*, it remains unclear if this would be beneficial or detrimental for the immune complex nephritis. However, as immune complex deposition was unaltered, angiotensin II-dependent changes in their handling remains only a remote possibility.

The greatest effect of the angiotensin II inhibition was observed in the glomeruli and consequently resulted in a significant reduction of proteinuria. A significant reduction of mesangial expansion could be observed in enalapril- or candesartan-treated mice as compared to vehicle or amlodipin-treated mice. RAS-inhibition reduced mesangial cell proliferation as shown by immunostaining for Ki-67, a cell proliferation marker. This observation opens the possibility for an additional, non-hemodynamic pathophysiological mechanism: angiotensin II could increase the glomerular expression of mediators such as growth factors, chemokines and cytokines. These mediators would modulate different processes, like the inflammatory cell recruitment or mesangial cell expansion.

Therefore we further analysed the expression of different chemokines. In a previous study we described that several chemokines (preferentially CCL2/MCP-1 and CCL5/RANTES) are up-regulated early in glomeruli of MRL/lpr mice (Pérez de Lema et al., 2001). Chemokines contribute to the progression of the disease by promoting the recruitment of leukocytes into the kidney. At the 14 weeks time point analysed, the major locations of CCL2/MCP-1 and CCL5/RANTES expression are the glomeruli (Pérez de Lema et al., 2001), which also corresponded to the sites of cellular infiltration and proliferation. According to our results, the blockade of angiotensin II differentially reduces the expression of some chemokine mRNA, such as CCL2/MCP-1, CXCL1/MIP-2 and CCL4/MIP-1ß. It has been described, that AT1-receptor antagonist treatment reduces CCL2/MCP-1 expression and monocyte/macrophage

infiltration in an anti-thymocyte serum induced glomerulonephritis (Wolf et al., 1998). ACE–inhibition also decreased recruitment of mononuclear cells in a rat model of immune complex nephritis through down-regulation of CCL2/MCP-1 and NF-κB inactivation (Ruiz-Ortega et al., 1998). Furthermore, treatments with ACEI or AT1 receptor antagonists reduced CCL2/MCP-1 mRNA synthesis in kidneys from rats with unilateral ureter ligation (Morrissey and Klahr, 1998).

Surprisingly, in our lupus-nephritis model CCL5/RANTES mRNA remained unaltered in enalapril or candesartan treatment groups, as compared to vehicle-treated mice. Wolf et al. found that angiotensin II can stimulate the expression of CCL5/RANTES both *in vitro* and *in vivo* (Wolf et al., 1997). Incubation of cultured rat glomerular endothelial cells or infusion of angiotensin II into rats resulted in an increase of CCL5/RANTES expression, an effect which was surprisingly mediated by AT2-receptors and independent of AT1 (Wolf et al., 1997). The unaltered mRNA levels for CCL5/RANTES in the present study remain unexplained at present.

Undisclosed is the question if chemokine expression is directly reduced by the RAS blockade or if the lower chemokine expression is an indirect effect of reduced inflammation. Effects of angiotensin II blockade on chemokine generation have been previously reported (Ruiz-Ortega et al., 1998; Wolf et al., 1998) but an intermediate role of other mediators of inflammation was not considered, although probably a network of different cytokines acting synergistically are involved.

Our results indicate, that angiotensin II inhibition has protective effects on the development of immune-mediated renal damage, an effect, which involves not only hemodynamic, but also immune-modulatory effects. We suggest that the antihypertensive effect of AT-1 blockers or ACEIs is an important but not exclusive mechanism by which the progression of renal disease is achieved. Through a reduction of chemokine expression, the inhibition of the RAS would

modulate the immune response in lupus nephritis and other nephropathies and thereby ameliorate the renal damage.

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References

Adachi M, Watanabe-Fukunaga R and Nagata S (1993) Aberrant transcription caused by the insertion of an early transposable element in an intron of the Fas antigen gene of lpr mice. *Proc Natl Acad Sci U S A* 90:1756-1760.

Bartels H, Bohmer M and Heierli C (1972) [Serum creatinine determination without protein precipitation]. *Clin Chim Acta* 37:193-197.

Burnier M and Brunner HR (2000) Angiotensin II receptor antagonists. *Lancet* 355:637-645.

Carvalho-Pinto CE, Garcia MI, Mellado M, Rodriguez-Frade JM, Martin-Caballero J, Flores J, Martinez AC and Balomenos D (2002) Autocrine production of IFN-gamma by macrophages controls their recruitment to kidney and the development of glomerulonephritis in MRL/lpr mice. *J Immunol* 169:1058-1067.

Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. *Anal Biochem* 162:156-159.

Herlitz H, Svalander C, Tarkowski A and Westberg G (1988) Effect of captopril on murine systemic lupus erythematosus disease. *J Hypertens Suppl* 6:S684-686.

Hisada Y, Sugaya T, Yamanouchi M, Uchida H, Fujimura H, Sakurai H, Fukamizu A and Murakami K (1999) Angiotensin II plays a pathogenic role in immune-mediated renal injury in mice. *J Clin Invest* 103:627-635.

Hoffmann JP (1971) [Stability of blood, serum and urine]. *Ther Umsch* 28:662-665.

Hollenberg NK (2000) Impact of angiotensin II on the kidney: does an angiotensin II receptor blocker make sense? *Am J Kidney Dis* 36:S18-23.

Kato S, Luyckx VA, Ots M, Lee KW, Ziai F, Troy JL, Brenner BM and MacKenzie HS (1999) Renin-angiotensin blockade lowers MCP-1 expression in diabetic rats. *Kidney Int* 56:1037-1048.

Kim S and Iwao H (2000) Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacol Rev* 52:11-34.

Lai KN, Leung JC, Lai KB, To WY, Yeung VT and Lai FM (1998) Gene expression of the renin-angiotensin system in human kidney. *J Hypertens* 16:91-102.

Luckow B, Maier H, Chilla S and Pérez de Lema G (2000) The mCK-5 multiprobe RNase protection assay kit can yield erroneous results for the murine chemokines IP-10 and MCP-1. *Anal Biochem* 286:193-197.

Mackenzie HS, Troy JL, Rennke HG and Brenner BM (1994) TCV 116 prevents progressive renal injury in rats with extensive renal mass ablation. *J Hypertens Suppl* 12:S11-16.

Mampaso FM and Wilson CB (1983) Characterization of inflammatory cells in autoimmune tubulointerstitial nephritis in rats. *Kidney Int* 23:448-457.

Merino R, Iwamoto M, Fossati L and Izui S (1993) Polyclonal B cell activation arises from different mechanisms in lupus- prone (NZB x NZW)F1 and MRL/MpJ-lpr/lpr mice. *J Immunol* 151:6509-6516.

Morrissey JJ and Klahr S (1998) Differential effects of ACE and AT1 receptor inhibition on chemoattractant and adhesion molecule synthesis. *Am J Physiol* 274:F580-586.

Nataraj C, Oliverio MI, Mannon RB, Mannon PJ, Audoly LP, Amuchastegui CS, Ruiz P, Smithies O and Coffman TM (1999) Angiotensin II regulates cellular immune responses through a calcineurin-dependent pathway. *J Clin Invest* 104:1693-1701.

Navar LG, Inscho EW, Majid SA, Imig JD, Harrison-Bernard LM and Mitchell KD (1996) Paracrine regulation of the renal microcirculation. *Physiol Rev* 76:425-536.

Pérez de Lema G, Maier H, Nieto E, Vielhauer V, Luckow B, Mampaso F and Schlondorff D (2001) Chemokine expression precedes inflammatory cell infiltration and chemokine receptor and cytokine expression during the initiation of murine lupus nephritis. *J Am Soc Nephrol* 12:1369-1382.

Pisetsky DS and Peters DV (1981) A simple enzyme-linked immunosorbent assay for antibodies to native DNA. *J Immunol Methods* 41:187-200.

Ravid M, Savin H, Jutrin I, Bental T, Katz B and Lishner M (1993) Long-term stabilizing effect of angiotensin-converting enzyme inhibition on plasma creatinine and on proteinuria in normotensive type II diabetic patients. *Ann Intern Med* 118:577-581.

Rovin BH, Rumancik M, Tan L and Dickerson J (1994) Glomerular expression of monocyte chemoattractant protein-1 in experimental and human glomerulonephritis. *Lab Invest* 71:536-542.

Ruiz-Ortega M, Bustos C, Hernandez-Presa MA, Lorenzo O, Plaza JJ and Egido J (1998) Angiotensin II participates in mononuclear cell recruitment in experimental immune complex nephritis through nuclear factor-kappa B activation and monocyte chemoattractant protein-1 synthesis. *J Immunol* 161:430-439.

Satoh M, Kashihara N, Yamasaki Y, Maruyama K, Okamoto K, Maeshima Y, Sugiyama H, Sugaya T, Murakami K and Makino H (2001) Renal interstitial fibrosis is reduced in angiotensin II type 1a receptor-deficient mice. *J Am Soc Nephrol* 12:317-325.

Scholzen T and Gerdes J (2000) The Ki-67 protein: from the known and the unknown. *J Cell Physiol* 182:311-322.

Singhal PC, Santiago A, Satriano J, Hays RM and Schlondorff D (1990) Effects of vasoactive agents on uptake of immunoglobulin G complexes by mesangial cells. *Am J Physiol* 258:F589-596.

Stauss HM, Godecke A, Mrowka R, Schrader J and Persson PB (1999) Enhanced blood pressure variability in eNOS knockout mice. *Hypertension* 33:1359-1363.

Tarkowski A, Carlsten H, Herlitz H and Westberg G (1990) Differential effects of captopril and enalapril, two angiotensin converting enzyme inhibitors, on immune reactivity in experimental lupus disease. *Agents Actions* 31:96-101.

Tesch GH, Maifert S, Schwarting A, Rollins BJ and Kelley VR (1999) Monocyte chemoattractant protein 1-dependent leukocytic infiltrates are responsible for autoimmune disease in MRL-Fas(lpr) mice. *J Exp Med* 190:1813-1824.

Theofilopoulos AN and Dixon FJ (1981) Etiopathogenesis of murine SLE. *Immunol Rev* 55:179-216.

Traynor TR and Schnermann J (1999) Renin-angiotensin system dependence of renal hemodynamics in mice. *J Am Soc Nephrol* 10 Suppl 11:S184-188.

Wada T, Yokoyama H, Su SB, Mukaida N, Iwano M, Dohi K, Takahashi Y, Sasaki T, Furuichi K, Segawa C, Hisada Y, Ohta S, Takasawa K, Kobayashi K and Matsushima K

(1996) Monitoring urinary levels of monocyte chemotactic and activating factor reflects disease activity of lupus nephritis. *Kidney Int* 49:761-767.

Wolf G, Schneider A, Helmchen U and Stahl RA (1998) AT1-receptor antagonists abolish glomerular MCP-1 expression in a model of mesangial proliferative glomerulonephritis. *Exp Nephrol* 6:112-120.

Wolf G, Ziyadeh FN, Thaiss F, Tomaszewski J, Caron RJ, Wenzel U, Zahner G, Helmchen U and Stahl RA (1997) Angiotensin II stimulates expression of the chemokine RANTES in rat glomerular endothelial cells. Role of the angiotensin type 2 receptor. *J Clin Invest* 100:1047-1058.

Zanchi A, Brunner HR, Waeber B and Burnier M (1995) Renal haemodynamic and protective effects of calcium antagonists in hypertension. *J Hypertens* 13:1363-1375.

Zoja C, Liu XH, Donadelli R, Abbate M, Testa D, Corna D, Taraboletti G, Vecchi A, Dong QG, Rollins BJ, Bertani T and Remuzzi G (1997) Renal expression of monocyte chemoattractant protein-1 in lupus autoimmune mice. *J Am Soc Nephrol* 8:720-729.

Footnotes:

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Figure 1: Effect of treatments on arterial blood pressure in MRL/lpr mice.

Mean arterial blood pressure was measured for 30 min -starting 90 min after antagonisation of anesthesia. All treatments, i.e. amlodipin, enalapril and candesartan, reduced blood pressure to a similar extent, as compared to the vehicle-treated group.

Values are means ± SD of 5 animals for each group. *$p<0.05$ vs. vehicle-treated animals.

Figure 2: Effect of treatments on plasma IgG and anti-DNA antibody concentrations in MRL/lpr mice.

(A) Plasma IgG levels, detected by ELISA, expressed in mg/ml. Enalapril- and candesartan-treated mice showed comparable circulating IgG levels as compared to vehicle- or amlodipin-treated mice. Values are means ± SD of 6 animals for each group (n=3 for Candesartan).

(B) Plasma anti-DNA antibody levels, detected by ELISA, expressed in µg/ml. Neither enalapril nor candesartan treatment had an effect on circulating anti-DNA antibody concentration as compared to amlodipin or vehicle-treated control groups. Values are means ± SD of 3-6 animals for each group.

Figure 3: Effect of treatments on renal morphology and immune complex deposition in MRL/lpr mice.

Light microscopy (100-400x) showing PAS stained renal tissue from representative 14 weeks-old mice after 6 weeks of treatment with either: vehicle (A), amlodipin (B), enalapril (C), candesartan (D). Enalapril or candesartan treated animals showed reduced glomerular damage, characterised by both reduced hypercellularity and mesangial matrix expansion. No changes were noted in the tubulointerstitial lesions in any group (here shown perivasculitis of vehicle (E) or candesartan (F) treated mice).

Fluorescence microscopy (200x) showing immunofluorescence-staining for IgG on renal tissue of representative 14 weeks-old mice treated with either: (G) vehicle, (H) enalapril (3.0 mg/kg) or (I) candesartan (5.0 mg/kg) for 6 weeks. Treatments showed no significant alteration of immune complex distribution or intensity of the staining.

Figure 4: Effect of treatments on glomerular damage in MRL/lpr mice.

(A) Albuminuria, detected by ELISA, expressed as µg albumin per mg urinary creatinine. Both, enalapril and candesartan treatment resulted in a significant reduction of urinary albumin excretion, as compared to vehicle-treated groups at 14 weeks of age. Urinary albumin in the amlodipin group was comparable to the vehicle-treated group. Values are means ± SD of 9-12 animals for each group. * $p<0.05$ vs. age matched vehicle-treated animals; § $p<0.05$ vs amlodipin-treated mice.

(B) Semi-quantitative analysis of glomerular injury, using the glomerular activity index. Values are means ± SD of 9-12 animals for each group. *$p<0.05$ vs. vehicle-treated animals; § $p<0.05$ vs amlodipin-treated mice.

(C) Quantitative analysis of Ki-67-positive cells, counted in 50 glomeruli of kidney sections from 4 mice per group. Values express mean positive cell numbers/glomerulus ± SD. *$p<0.05$ vs. vehicle-treated animals; § $p<0.05$ vs amlodipin-treated mice.

Figure 5: Effect of treatments on renal expression of chemokines in MRL/lpr mice.

Densitometric analysis of chemokine mRNA expression, detected by ribonuclease-protection assay, expressed as the chemokine to GAPDH expression ratio in arbitrary units. Value 1.0 corresponds to the mean of each chemokine in vehicle-treated mice. Both, enalapril or candesartan treatment reduced the expression of CCL2/MCP-1, CCL4/MIP-1ß and

CXCL1/MIP-2 by 50-70 % as compared to vehicle treatment. By contrast, CCL5/RANTES expression remained unaffected by RAS inhibition. Values are means ± SD of 4 animals for each group. *$p<0.05$ vs. vehicle-treated animals.

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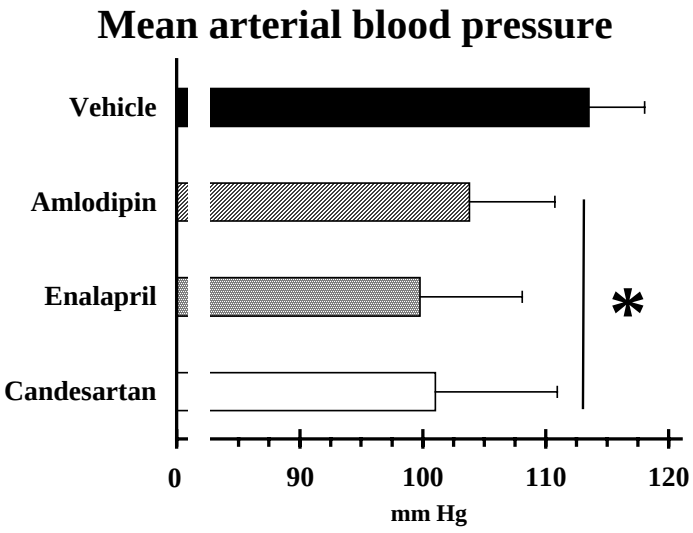

Figure 1

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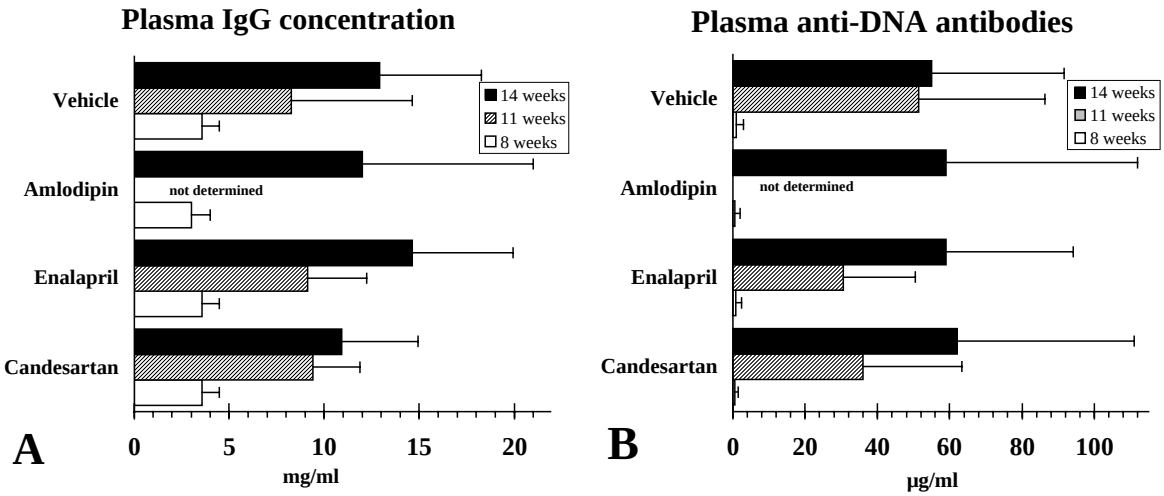

Figure 2

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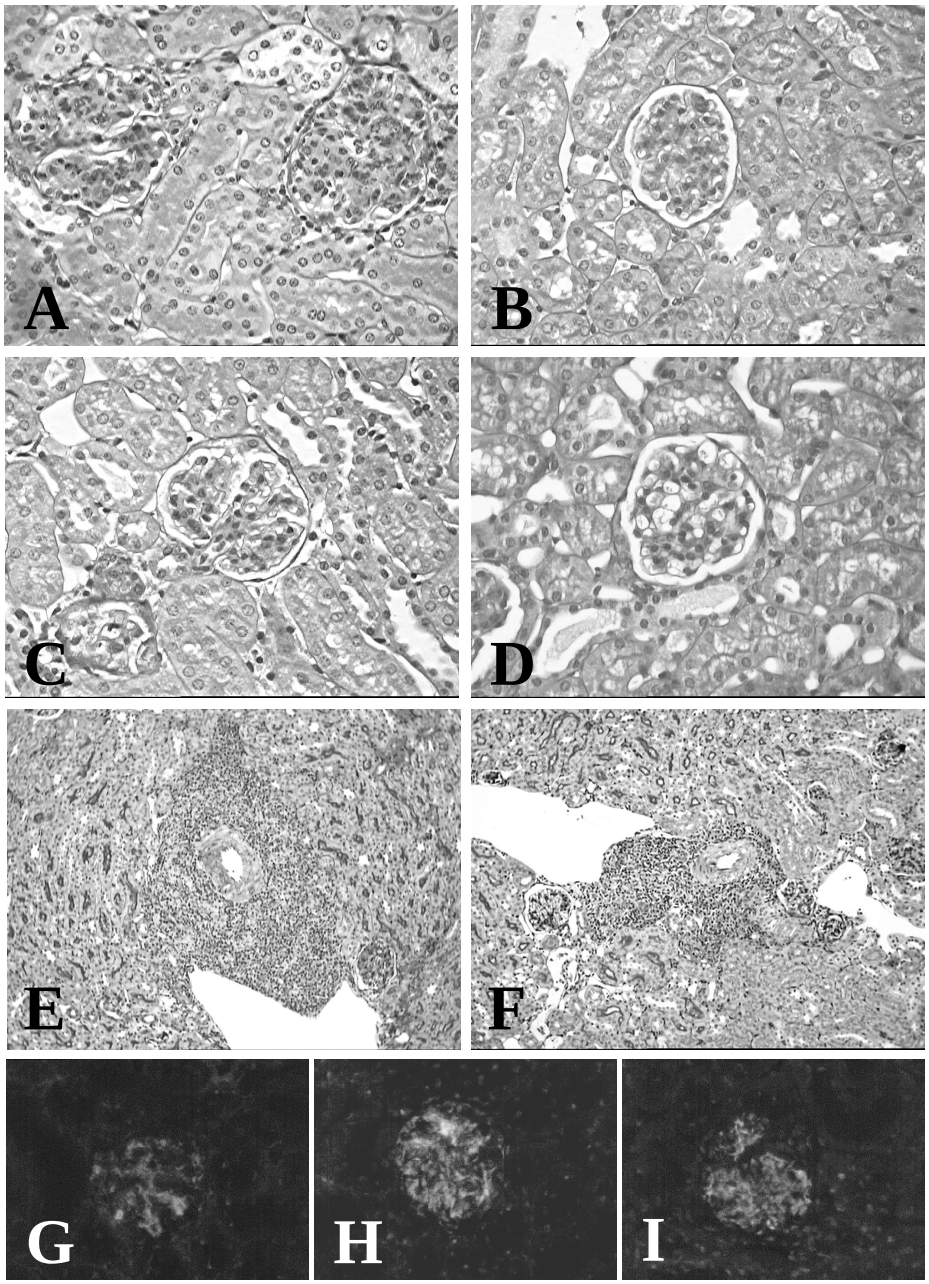

Figure 3

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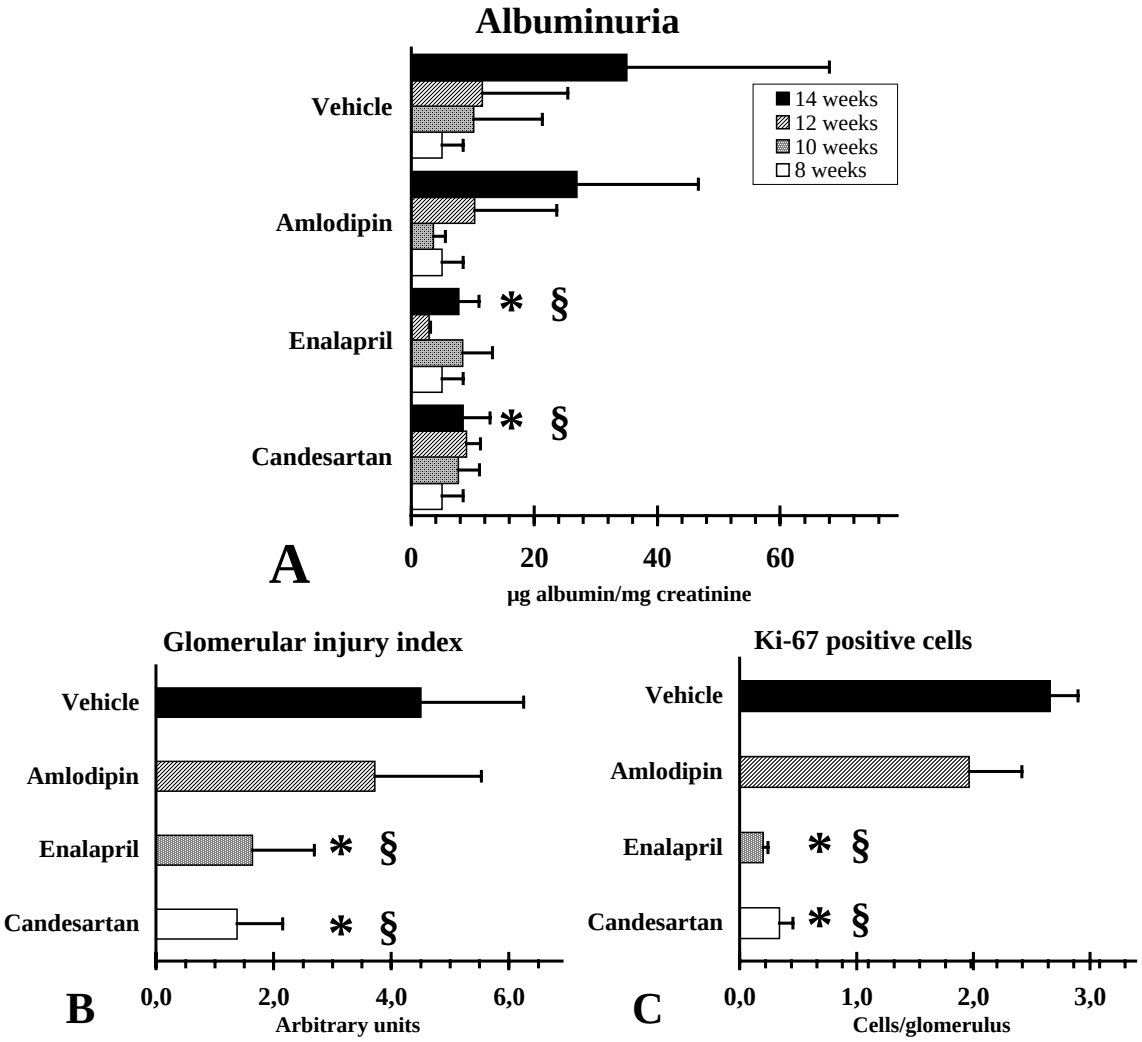

Figure 4

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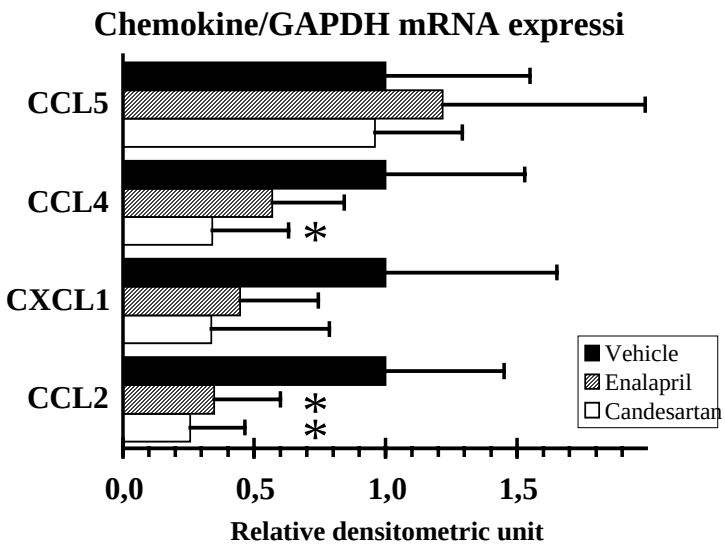

Figure 5